REINFORCEMENT AND CASCADE REINFORCEMENT IN THE *LUCANIA* SYSTEM: THE EFFECTS OF EXPERIMENTAL DESIGN, SEX, AND HETEROSPECIFIC PAIRINGS ON MATE PREFERENCE

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THESIS

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ABSTRACT

Reinforcement and cascade reinforcement are potentially very potent evolutionary forces (Butlin 1987; Servedio and Noor 2003; Fuller 2016; Pfennig 2016). Their pervasiveness in nature, however, can only be determined through documentation. Currently, the only way to document these processes is to compare levels of reproductive isolation between areas of sympatry and allopatry (Servedio and Noor 2003; Hoskin and Higgie 2010; Pfennig 2016). This often involves using behavioral assays and metrics to determine conspecific or native mate preference in the laboratory. Despite the importance of using assays and metrics that correctly detect reproductive isolation, studies often do not test whether their experimental design accurately measures mate preference. Here, I aimed to determine the best way to measure reproductive isolation using the *Lucania* (*Lucania goodei* and *Lucania parva*) system as a model organism. In my first experiment, I tested multiple assays and metrics of behavior to determine which most accurately measured conspecific and native mate preference for male and female *L. goodei*. I found that measurements of mating behaviors (i.e. egg production and courting behavior) reliably detected mate preference for male and female *L. goodei,* while measurements of association time failed to do the same. I also found that only female *L. goodei* exhibited native mate preference. In my second experiment, I investigated whether previous estimates of reproductive isolation inflated sympatric estimates due to their limited heterospecific pairings. I found that reproductive isolation in male sympatric *L. parva* is far weaker than previously estimated. Ultimately, I highlight the importance of: using appropriate behavioral assays and metrics to determine reproductive isolation, using both sympatric and allopatric heterospecific stimulus mates when determining levels of reproductive isolation, and measuring reproductive isolation in both sexes.

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CHAPTER 1: INTRODUCTION

The biological species concept offers one solution (of many) to the age-old question of what makes a "good species"(Mayr 1942; de Queiroz 2005). The main criterion of the biological species concept is the presence of reproductive isolation between groups (Mayr 1942; Coyne 2004). Reproductive isolation, however, often arises as an incidental effect of selection on other ecologically relevant traits (Coyne 2004). This means that species diverging in allopatry may come back into secondary contact before reproductive isolation has formed. Without reproductive isolation in place, species in secondary contact may run the risk of hybridization or collapse (Servedio and Noor 2003; Coyne 2004; Abbott et al. 2013). Luckily, a single form of selection, called reinforcement, can directly increase reproductive isolation between groups in sympatry and complete speciation (Butlin 1987; Servedio and Noor 2003; Ortiz-Barrientos et al. 2009). Reinforcement acts by selecting against unfit hybrids (Servedio and Noor 2003; Saetre 2012). In turn, selection favors preferences and traits that increase accuracy of species recognition eventually completing speciation. Reinforcement may not only be responsible for completing speciation, but may also initiate it. If traits or preferences in sympatry change enough, species may begin to discriminate against conspecific mates from foreign populations. This increased native preference in sympatry is called cascade reinforcement (Ortiz-Barrientos et al. 2009; Pfennig 2016). Cascade reinforcement is particularly interesting, because it can initiate speciation in the absence of post-zygotic isolation, a deed previously attributed only to sexual selection (Hoskin and Higgie 2010; Comeault and Matute 2016). Needless to say, reinforcement and cascade reinforcement are potentially very potent evolutionary forces.

Initially, the idea of reinforcement was met with skepticism (Noor 1999; Marshall et al. 2002). Theoretical and limited empirical evidence, however, eventually showed that the process was feasible (Coyne and Orr 1989; Liou and Price 1994). Despite this breakthrough, the dearth of empirical evidence made it hard to gauge the pervasiveness of reinforcement in nature. To determine the frequency of reinforcement, studies began to document its occurrence. To do this, studies measured and compared levels of reproductive isolation between species found in sympatry and allopatry. Increased reproductive isolation in sympatry compared to allopatry is a hallmark of reinforcement and is termed reproductive character displacement (Servedio and Noor 2003; Hoskin and Higgie 2010). Documenting reproductive character displacement between populations allowed empirical evidence for reinforcement to grow significantly (Sætre et al. 1997; Pfennig and Simovich 2002; Nosil and Yukilevich 2008; Lemmon 2009). Likewise, a similar method of comparing native mate preference between populations is now used to determine how often reinforcement leads to cascade reinforcement, although this is still relatively unknown (Comeault and Matute 2016; but see: Kozak et al. 2015). Ultimately, detecting reinforcement and cascade reinforcement hinges upon measuring reproductive isolation between groups. The best way to measure reinforced reproductive isolation, however, remains relatively untested (but see: Wagner 1998; Dougherty and Shuker 2015).

My goal here is to help determine the best way to measure reproductive isolation in the *Lucania* system to identify reinforcement and cascade reinforcement. In my first experiment, I used the bluefin killifish (*Lucania goodei)* to determine which behavioral assays and metrics best detect conspecific and native mate preference for males and females. Contrary to the findings of previous studies (Cummings and Mollaghan 2006), I found that mating behavior metrics (such as the

number of eggs produced or courting behavior) most reliably detected mate preference in both sexes of *L. goodei*, while the association time metric failed to do so. Testing preferences in both sexes using multiple assays and measurements allowed me to also determine that female *L. goodei* have stronger conspecific mate preferences than male *L. goodei,* and that they are the only group to exhibit native mate preference.

In my second experiment, I ask if the limited heterospecific pairings from previous studies inflated reproductive isolation for sympatric groups. Typical experiments measure and compare reproductive isolation between heterospecifics from sympatric populations and heterospecifics from allopatric populations (hereafter referred to as traditional pairings). But, they typically do not pair individuals from sympatric populations with individuals from allopatric populations. To determine if reproductive isolation was previously inflated, I paired bluefin and rainwater killifish (*Lucania goodei* and *Lucania parva)* from sympatric and allopatric populations and measured the levels of reproductive isolation. When I compared these levels of reproductive isolation to levels calculated using traditional parings I found that male sympatric *L. parva* had much weaker conspecific mate preferences than previously thought. I further investigated if males and females varied in their preference for native mates, and found that only female sympatric *L. parva* exhibited this preference.

In conclusion, the results of my experiments provide new information on how best to measure reproductive isolation in the *Lucania* system, and thus reinforcement and cascade reinforcement. First, I found that using particular assays and measurements are important for accurately estimating conspecific and native mate preference. Second, I found that pairing sympatric groups

with allopatric groups produces more accurate measurements of reproductive isolation. Finally, I noted the importance of measuring reproductive isolation for both sexes.

CHAPTER 2: MEASURING CONSPECIFIC AND NATIVE MATE PREFERENCES IN *LUCANIA GOODEI:* HOW EXPERIMENTAL DESIGN AFFECTS MATE PREFERENCE

INTRODUCTION

Many questions in evolutionary biology require the measurement of animal mate preference. Accurately determining mate preference in the laboratory, however, is quite challenging. Typically, measuring mate preference in the laboratory is comprised of two components: 1) how a focal individual is presented with stimulus mates (hereafter referred to as the 'assay'), and 2) the behaviors or actions that are measured during the assay as a proxy for mate preference (hereafter referred to as the 'metric'). Generally, behavioral assays can be broken into two categories: no-choice assays and choice assays (Dougherty and Shuker 2015). While choice assays present a focal individual with two or more potential mates, no-choice assays present a focal individual with only a single potential mate (Rundle and Schluter 1998; Wagner 1998; McGhee et al. 2007; Nosil 2007; Dougherty and Shuker 2015). Metrics also vary, and can range from condition-dependent behaviors, such as the frequency of courting bouts, to condition-independent behaviors, such as association time (Hunt et al. 2005; Cotton et al. 2006; Cummings and Mollaghan 2006). An assay or metric's ability to accurately detect mate preference, however, can depend on many factors.

When using assays and metrics, studies assume that they reliably detect the correct mate preference, and that they detect said mate preference to the appropriate magnitude. An assay or metric's ability to do this, however, can depend on whether organisms naturally encounter mates

concurrently or sequentially (Dougherty and Shuker 2015), whether mate preference is condition-dependent (Hunt et al. 2005; Cotton et al. 2006), or even the strength of mate preference (Houde 1997; Coyne 2004). Dougherty and Shuker (2015) found that measuring mate preference for the same species using multiple assays often resulted in differing reports of mate preference, with choice assays consistently detecting stronger mate preferences than no-choice assays. Furthermore, a single assay can vary in its ability to detect preference depending on the sex of the organism or whether preference is at the between- or within-species level (Dougherty and Shuker 2015). Metrics can similarly vary in their ability to reliably detect mate preference. For example, Cummings and Mollaghan (2006) measured female northern swordtail (*Xiphophorus nigrensis*) mate preference using two metrics: glides (a mating behavior) and association time. They found, however, that only association time was repeatable across several days. Clearly, methodology for measuring mate preference is highly variable, but few studies verify that their assays or metrics reliably detect mate preference.

Despite the fact that most studies do not investigate the reliability of the assays or metrics used to detect mate preference, a few studies do give general advice for reliably detecting mate preference. First, Wagner (1998) suggests that measuring an individual's mate preference several times provides the best estimate of their mate preference. Second, Dougherty and Shuker (2015) suggest using several different assays to measure mate preference, as similar results will corroborate any findings. While these approaches are warranted for exploring unknown mate preferences, I suggest that organisms with known preferences should be used as a control for testing the reliability of assays and metrics.

Here, I aim to use the above techniques to determine which assays and metrics most reliably detect mate preference in the bluefin killifish (*Lucania goodei)*. *L. goodei* is an excellent species to test the reliability of behavioral assays and metrics because they have strong and well documented conspecific mate preference when found in sympatry with their sister species, *L. parva* (Fuller et al. 2007; Fuller 2008a; Berdan and Fuller 2012; Gregorio et al. 2012; Kozak et al. 2015). In sympatry, *L. goodei* and *L. parva* mate and produce unfit hybrids at low levels (Walker and Johnson 1943). Selection against hybridization favors increased conspecific mate preference (pre-zygotic isolation/behavioral isolation) in a phenomenon termed reinforcement. Reinforcement is potentially a very potent evolutionary force, because it is the only form of selection that can *directly* complete the speciation process between groups. While initially met with skepticism, reinforcement is now widely accepted and has been documented in the *Lucania* system as well as several other species (Birds: Sætre et al., 1997; Frogs: Blair, 1974; Lemmon, 2009; Fish: Fuller et al., 2007; Plants: Matute and Ortiz-Barrientos, 2014 ; Insects: Nosil, 2007, Kelly and Noor, 1996, Yukilevich & True, 2006). The effects of reinforcement in the *Lucania* system not only makes *L. goodei* an ideal study organism for testing the reliability of metrics and assays, but also allows me to test two additional implications of reinforcement.

First, reinforcement predicts that the increase in pre-zygotic isolation should coincide with increased costs of hybridization (Yukilevich 2012). This means that asymmetric hybrid fitness, between species or even between sexes, should lead to asymmetric pre-zygotic isolation (Bolnick et al. 2008; Hoffmann and Turelli 1997; Jaen ike et al. 2006; Pfennig and Simovich 2002). As an example, consider two species A and B where hybrids formed by matings between A females and B males have lower fitness than the reciprocal hybrid (B females x A males). Reinforcement

may result in a scenario where A females and B males are less likely to hybridize compared to B females and A males. In fact, Yukilevich (2012) found good evidence for such a pattern in *Drosophila*. Another possibility, however, is that females should always have higher levels of behavioral isolation than males. In general, females invest more than males in a given reproductive event, particularly in systems where males do not provided parental care (Wirtz 1999; Coyne 2004; Yukilevich 2012).

Lucania is a good system to test these scenarios because there are asymmetric fitness costs to hybridization for male and female *L. goodei* and *L. parva*. Crosses between *L. goodei* females and *L. parva* males produce F1 hybrid offspring with no discernible decrease in fitness (Fuller 2008a). On the other hand, crosses between *L. goodei* males and *L. parva* females produce F1 males whose fertilization success is reduced by at least 50% (Fuller 2008a). The hypothesis that asymmetries in hybrid fitness should be reflected in the strength of behavioral isolation predicts that *L. goodei* males should have high levels of preference for conspecifics. In contrast, the hypothesis that females energetically invest more into a given reproductive event (and that a given mating event is cheap for males) predicts that females should have high levels of conspecific preference.

The second implication is that reinforcement can lead to correlated effects on within-species preferences. Reinforcement's signature is shifted mating traits and preferences in areas of sympatry compared to allopatry. If traits and preferences shift drastically, individuals from sympatric populations may begin to discriminate against conspecific mates from foreign populations. This increase in native mate preference as an incidental effect of reinforcement is

known as cascade reinforcement (Ortiz-Barrientos et al. 2009; Fuller 2016; Pfennig 2016). Cascade reinforcement can theoretically result in rapid diversification of mating traits and preferences leading to speciation events in the absence of post-zygotic isolation (a concept previously attributed only to sexual selection) (Hoskin and Higgie 2010). How frequently cascade reinforcement occurs in nature, however, is still largely unknown. Cascade reinforcement has been documented in *L. parva*. Kozak et al. (2015) found that sympatric female *L. parva* preferred native mates significantly more than foreign mates, while allopatric female *L. parva* showed no preference. Whether cascade reinforcement is also present in *L. goodei* is unknown.

In summary, my experiment had three goals. The first goal was to determine which behavioral assays or metrics reliably detect *L. goodei* mate preference. The well documented effects of reinforcement in the *Lucania* system led me to consider assays or metrics to be reliable if they detected conspecific mate preference for *L. goodei*, and if the strength of conspecific preference roughly agreed with the estimates of other assays or metrics within this study and with the estimates of strength of preference from the literature. The second goal was to use multiple assays and metrics to determine if *L. goodei* have native mate preferences consistent with cascade reinforcement. Finally, my third goal was to use multiple assays and metrics to determine if mate preference (either at the between- or within-species level) varies between sexes. Using multiple assays and metrics for the final two goals allowed me to corroborate my mate preference findings. I measured conspecific and native mate preference for males and females using three different assays and three different metrics of preference.

METHODS

Collection and Care

I needed three types of populations for my experiment: 1) a focal population of *L. goodei*, to measure mate preference, 2) a heterospecific population of a closely related species to be used as stimulus mates, and 3) a second, distinct population of *L. goodei,* to be used as foreign stimulus mates. I collected my focal population of *L. goodei* from the Lower Bridge of the Wakulla River in northern Florida (Wakulla County, Florida). Previous studies have repeatedly shown high levels of conspecific preference in this population (Fuller 2008a; Gregorio et al. 2012; Kozak et al. 2015). I also collected heterospecific stimulus mates (*L. parva)* from this same population. Finally, I collected foreign *L. goodei* stimulus mates from Blue Springs, Florida (Gilchrist County, Florida), a site that occurs in a separate drainage (Suwanee) and that differs in mtDNA sequence (Murphy, unpublished data). I used dip nets and seines to collect at least 10 males and 10 females from each population during the summers of 2015 and 2016. Fish were transported in coolers back to the University of Illinois, where they were housed in stock tanks in a greenhouse. Fish were exposed to natural light cycles, and fed a diet of brine fish and blood worms daily.

Administration of Assays

I randomly selected 10 male and 10 female *L. goodei* individuals from the Lower Bridge population to be used as focal individuals for this study. Each focal individual experienced two rounds of testing. The first round sought to measure conspecific mate preference, while the second round sought to measure native versus foreign mate preference. Each round of testing occurred over a period of nine days. Identical methods were used with the exception of the identity of the stimulus animals (conspecific versus heterospecific; native versus foreign). On

day one, focal individuals took part in a dichotomous choice assay immediately followed by an audience assay. On day two, both assays were administered a second time. Once assays were completed on day two, a stimulus mate was randomly assigned to remain with the focal individual. The pair was given 24 hours to acclimate to the tank and then no-choice assay began.no-choice assays lasted for seven days and eggs were collected and counted from each pair daily. At the end of this first round of testing, stimulus mates were removed and focal individuals were given a rest period of several days. After the rest period, the above process was repeated using native and foreign stimulus mates.

Dichotomous Choice Assay: This assay involves a free swimming focal individual (either a male or female *L. goodei*) choosing between two caged stimulus mates. Focal individuals were placed into 38-liter tanks at least 24-hours prior to the experiment. Immediately before the start of the experiment, I placed two mesh cages ½ inch below the tank waterline in the front two corners of the tank. I randomly assigned and placed stimulus mates into the cages and gave them 10 minutes to acclimate to the new environment. After the acclimation period, I allowed the cages to rest on the bottom of the tank, about an inch away from the corners. Moving the cages gave the focal individual freedom to approach stimulus mates from above and from all four sides. Once stimulus mate cages rested on the bottom of the tank, I began the assay which lasted for 10-minutes. During the assay, I recorded the amount of time focal individuals spent within one body length of each stimulus mate and the number of courting bouts performed by males. I measured courting bouts for both the focal males (i.e., males choosing among females) and the stimulus males (i.e., males being chosen by females). This assay was repeated on days one and two.

From these data, I calculated conspecific and native preference for both males and females using both time associated with each stimulus mate and courting bouts given to each stimulus mate (males) or received by focal individuals (females). I first calculated time-preference as the signed difference in association time between conspecifics and heterospecifics (i.e. time spent with conspecifics minus time spent with heterospecifics) for each focal individual for each day. Likewise, I calculated courtship-preference as the difference in courtship bouts (either given or received) between conspecifics and heterospecifics for each focal individual for each day. Assays where focal individuals spent no time with stimulus mates or where no courting was performed were not considered in analysis (adjusted sample sizes reflected in tables 2.2 & 2.3). I next asked whether preferences differed between days one and two. I found no differences, and I subsequently summed the association times and the courtship bouts and then calculated their signed differences. The same metrics were calculated for native versus foreign assays. Positive values indicate preference for conspecific (or native) mates, whereas negative values indicate preference for heterospecific (or foreign) mates. I tested whether preferences differed from a null expectation of zero (no preference) using a one-sample t-test.

Audience Assay: Audience assays provided the focal individual the choice between a restrained mate and a free-swimming mate. The audience assays allowed males and females to interact in a natural fashion while preventing competition among stimulus mates. By having an alternate, caged stimulus present, the audience assay also provided a potential comparison to the freely available mate. Such a comparison is absent in the no-choice assays.

At the end of the dichotomous choice assays, two restrained stimulus mates rested on the bottom of the tank in their respective cages. Following a dichotomous choice assay, one of the stimulus mates was randomly chosen to be released. Once free, the empty cage was removed from the tank and the audience assay began. Assays lasted for 10-minutes. During the assay, I recorded the amount of time the focal individual spent within one body length of each stimulus mate (either caged or free) and the number of courting bouts performed by males (either as stimulus mates or as focal individuals). I repeated this assay on day two, but reversed which mate was released. On day two, the caged stimulus mate from day one was freed, and the free stimulus mate from day one was caged.

The statistical methods used to measure preference for conspecific and native mates was identical to that used in the dichotomous choice assays. I summed the amount of time spent with conspecifics and with heterospecifics (regardless of whether they were free or caged) across the two days and calculated their signed difference. Likewise, I summed the amount of courtship given (males) or received (females) across the two days and calculated the difference between conspecific and heterospecific. Assays where focal individuals spent no time with stimulus mates or where no courting was performed were not considered in analysis (adjusted sample sizes reflected in tables 2.2 & 2.3). Qualitatively identical results were obtained when I compared preference for free mates (i.e. time spent with free conspecific versus free heterospecific on separate days) or when I compared preference for caged mates (i.e. time spent with caged conspecific versus caged heterospecific on separate days). I used the same statistical methods to measure preferences for native versus foreign mates. I tested whether preferences differed from a null expectation of zero using a one-sample t-test.

No-Choice Assay: No-choice assays compared the total number of eggs produced between conspecific and heterospecific mate pairs. No-choice assays did not provide the focal individuals a choice between mates and instead paired them with a single mate for seven days. For conspecific preference trials, I randomly assigned either a conspecific or heterospecific stimulus mate to each focal fish. Likewise, for native versus foreign preference trials, I randomly assigned either a native or foreign stimulus mate to each focal fish. The mate was placed into the tank along with two top and two bottom yarn mops. The mops provided spawning substrate for the fish. The 'top mops' were attached to Styrofoam balls to allow them to float at the top of the tank, while the 'bottom mops' were attached to PVC pipe and laid on the bottom of the tank. After the stimulus mate was added to the tank, the pair was given 24-hours to acclimate. After the acclimation period, I collected and counted eggs from the mops each morning for seven days. At the end of the assay period, stimulus mates were removed from the tank and returned to stock tanks.

Here, I simply measured preference as the total number of eggs spawned. For each sex, I tested whether there was a difference between the number of eggs laid with conspecifics versus heterospecifics or between native versus foreign mates using either a two-sample t-test or a Kruskal-Wallis non-parametric test. I used the Kruskal-Wallis test when there were zero eggs across all trials with heterospecifics, thus violating the assumptions of the parametric t-test. All statistical tests were two-tailed. All analyses were performed in R (version 1.0.136).

Quantifying Reproductive Isolation

For all three assays, I calculated reproductive isolation (RI) for each assay and metric.

To calculate RI, I used Stalker's (1942) equation for reproductive isolation:

$$\frac{(Conspecific\ metric - Heterospecific\ metric)}{(Conspecific\ metric + Heterospecific\ metric)}$$

This equation creates a relative measure of preference, allowing for the direct comparison of the different metrics. The measure of RI ranges from -1 to 1, with negative numbers representing heterospecific (or foreign) preferences and positive numbers representing conspecific (or native) preferences. For the time and courting metrics for both the dichotomous choice and audience assays, I could calculate RI for each individual and calculate the 95% confidence intervals around the population mean. However, I could not directly apply this formula to individuals in my no-choice data since focal individuals were only exposed to one mate. Instead, I used a bootstrap resampling method to determine mean RI and 95% confidence interval for each sex (5,000 replicates). The population level RI was calculated as the scaled difference in the number of eggs laid with conspecific (or native) versus heterospecific (or foreign) mates. I calculated RI for each of the 5,000 replicates and used these to calculate the 95% confidence interval.

Finally, I used Stalker's formula to calculate RI values for *Lucania* using data from previous studies (Table 2.1). I used these RI values as a baseline to evaluate my newly calculated RI values. In addition to the criteria that reliable metrics and assays would detect a statistically significant preference for conspecific mates and that they would roughly agree with one another, I included the third criterion that reliable metrics and assays would have RI values consistent with those from previous studies.

RESULTS

Determining Reliable Assays and Metrics

The first goal of this experiment was to determine which assays and metrics reliably detect conspecific mate preference for *L. goodei*. I determined that metrics and assays would need to meet the following criteria to be considered reliable: First, they would need to detect a statistically significant conspecific mate preference for both males and females. Second, RI calculations for assays and metrics should be consistent with one another as well as consistent with previous studies' estimates. Using these criteria, I found that all assays detected male conspecific mate preference, although one was only marginally significant (Figure 2.1, Table 2.2). Males spent significantly more time with conspecific mates and courted them more often during dichotomous choice assays (Figure 2.1A& 2.1B). The RI values for dichotomous choice metrics were also consistent with one another (RI-time = 0.71, RI-courting = 0.81), and their 95% confidence intervals overlap (Table 2.2). During audience assays, males courted conspecific mates significantly more often, but only spent marginally more time with them (Figure 2.1A& 2.1B). RI values for both metrics exceeded RI values calculated from previous studies, but were lower than dichotomous choice RI values (Table 2.2). Finally, no-choice assays also detected male mate preference as assays with conspecific pairs produced significantly more eggs than assays with heterospecific pairs (Figure 2.1C, Table 2.2). Furthermore, the RI value for no-choice assays was similar to those found in the audience assay (~0.5) (Table 2.2).

Assays and metrics varied much more in their ability to detect female mate preference. During dichotomous choice assays, females did not prefer to spend time with one mate over the other (Figure 2.2A). Relatively few courting bouts occurred in the female dichotomous choice assays, but the courtship that females did receive was solely from conspecifics (Figure 2.2B). The value for RI clearly varied between the two metrics. The RI for the time metric severely

underestimated female mate preference (RI-time=0.02), while the RI for the courting metric reported significant conspecific preference (RI-courting=1.00) (Table 2.2). Audience assays did detect female conspecific mate preference. Females spent significantly more time with and were courted more often by conspecific mates (Figure 2.2A& 2.2B). However, the RI value for courting was nearly twice as high as the RI value for time in the audience assays (Table 2.2). Finally, the no-choice assay could also detect conspecific mate preference. Females produced significantly more eggs with conspecific partners than with heterospecific partners (Figure 2.2C). In fact, zero eggs were produced during the entirety of the assay period when female *L. goodei* were paired with heterospecific mates. Since females laid zero eggs with heterospecifics, RI for the no-choice assays was very high (RI-eggs=1.00) (Table 2.2). The RI values for courting in the dichotomous choice and audience assays and the RI values for the no-choice assays were all similar to one another.

Native vs Foreign Mate Preference

The second goal of this experiment was to use several metrics and assays to determine if Lower Bridge *L. goodei* preferred native or foreign mates. I first looked for mate preference in Lower Bridge males, but found that none of my assays or metrics detected a significant preference for either mate (Figure 2.3). My RI calculations also reflected this lack of preference (Table 2.3). The RI values of two metrics, however, did stand out. The courting metric from the dichotomous choice assays and the total number of eggs produced metrics from the no-choice assays detected the strongest mate preference in males (RI-courting=-0.37 and RI-eggs=-0.17) (Table 2.3). Remarkably, both RI calculations indicate that males may prefer foreign mates. Next, I looked at native or foreign mate preference in Lower Bridge females. Here, I did detect a significant

preference for native mates. Females were courted significantly more often by native mates during dichotomous choice assays (Figure 2.4B). They also spent more time with and were courted more often by native mates during audience assays (Figure 2.4A& 2.4B). RI values for each of these metrics also matched this pattern (0.66, 0.64, 0.89, respectively) (Table 2.3). The RI value for the no-choice assay was 0.34 and was not statistically different from zero, but there was a large outlier (Figure 2.4C). Removal of this data point results in a significant preference for native males. Overall, only Lower Bridge females appear to have a within-species mate preference for native mates.

Male and Female Comparison

While Lower Bridge males and females both preferred conspecific mates, female preference was stronger than male preference. Female mate preference was stronger for: 1) the courting metric in the dichotomous choice assay, 2) the courting metric in audience assays, and 3) the total number of eggs produced metric in no-choice assays (Table 2.2). RI values for these metrics were not only higher than male RI values, but they were also similar to one another (Table 2.2). The time metric was the most inconsistent mate preference measurement between males and females. In the dichotomous choice assay, male RI was significantly higher than female RI, but the audience assay detected no difference between the sexes (Table 2.2).

Native mate preference also differed between male and female *L. goodei*. I observed native mate preference for female *L. goodei,* but found little evidence of the same preference for males. For females, both time and courtship in the audience assays, and courtship received in the dichotomous choice assay revealed high RI values with overlapping 95% confidence limits

(Table 2.3). The time metric in the dichotomous choice assay again underestimated RI values for females. For males, all metrics showed little preference for native females, and, if anything, revealed a slight preference for foreign mates (Table 2.3).

DISCUSSION

The first goal of this study was to determine which metrics or assays reliably detected *L. goodei* mate preference. Ultimately, I identified two metrics and assays that met my reliability criteria. First, the courting metric from audience assays detected that both males and females courted or were courted by conspecific mates more often than heterospecific mates (fulfilling criteria 1; Figure 2.1B & 2.2B). The RI values for both males and females were also roughly consistent with those found using other assays and metrics in this study, as well as consistent with previous estimates in the literature (fulfilling criteria 2; Table 2.2). The second reliable metric was the total number of eggs produced from the no-choice assay. Conspecific pairings produced significantly more eggs than heterospecific pairings for both males and females (fulfilling criteria 1; Figure 2.1C & 2.2C). Additionally, the RI values for both males and females were roughly consistent with those found using other assays and metrics in this study, as well as consistent with previous estimates in the literature (fulfilling criteria 2; Table 2.2). The RI values from courting metric and the total number of eggs produced metric were also consistent with one another for both males and females (fulfilling criteria 2; Table 2.2). These results were somewhat surprising. Previous studies concluded that association time predicted mate preference more reliably than metrics specifically measuring mating behaviors, such as gliding (Cummings and Mollaghan 2006). Cummings and Mollaghan (2006) argued that association time was more reliable because individuals could associate with a preferred mate regardless of breeding

condition. While this may be true for organisms who only approach or spend time with desired mates, it does not fit with the *L. goodei* mating ritual. Male *L. goodei* establish territories, while female *L. goodei* sequentially visit said territories (Fuller 2001, 2008b; McGhee et al. 2007). A female may visit, and thus spend time with, a male who she ultimately does not mate with (Fuller 2001). Sexes may also associate for reasons unrelated to reproduction, such as schooling. How *L. goodei* naturally encounters mates may factor in to why association time was such a poor indicator of preference.

The second goal of this study was to determine if *L. goodei* from the Lower Bridge population had native or foreign mate preferences. I found that female *L. goodei* preferred native mates, while male *L. goodei* showed no mate preference (Table 2.3). Native mate preference in female *L. goodei* is not only consistent with the prediction that reinforcement can cascade into within-species preferences, but also mirrors results from previous studies. Kozak et al. (2015) found that female *L. parva* exhibited native mate preference, while male *L. parva* showed no preference for native or foreign mates. Although this finding is a first step in determining if cascade reinforcement is occurring in *L. goodei,* it does not confirm cascade reinforcement per se. The signature of cascade reinforcement is strengthened native mate preferences in sympatry compared to allopatry (Ortiz-Barrientos et al. 2009). Future studies comparing female native mate preferences in sympatry and allopatry are needed to confirm cascade reinforcement in *L. goodei*.

Finally, the last goal of this study was to determine if female and male *L. goodei* mate preference differed. I found that *L. goodei* females had stronger mate preference than males. While both

sexes exhibited a significant preference for conspecific mates, RI values for females were consistently ~0.98 while RI values for males was closer to ~0.55 (Table 2.2). Female *L. goodei* were also the only sex to exhibit significant native mate preference (Table 2.3). The stronger mate preference of female *L. goodei* supports the hypothesis that larger energetic investment in reproduction by females drives asymmetric pre-zygotic isolation in the *Lucania* system. This was unexpected, because hybrid offspring with *L. goodei* fathers (and *L. parva* mothers) suffer higher fitness costs than hybrid offspring with *L. goodei* mothers (and *L. parva* fathers*)* (Fuller 2008b). It appears that the amount of energy invested by females in reproduction, and is therefore potentially lost during heterospecific pairing, outweighs the costs that males incur from unfit offspring. Differential investment in reproduction between sexes is well-documented, but this study provides early support for how reinforcement subsequently acts on the differences between sexes (Livingstone 1974; Wigby and Chapman 2005; Hayward and Gillooly 2011; Rankin et al. 2011).

In conclusion, I found that *L. goodei* mate preference was best detected using mating behaviors, such as egg production or courting. Using these metrics, I supported the finding of conspecific mate preference in sympatric *L. goodei*. I added further to this, by documenting differences in the strength of mate preference between sexes, where female *L. goodei* had much stronger conspecific mate preference than males. Not only did females have stronger conspecific mate preference, but they were also the only sex to exhibit native mate preference. My findings provide support for the hypothesis that differential investment in reproductive events can affect the formation of pre-zygotic isolation via reinforcement. Ultimately, my study adds to research

determining the best ways to measure mate preference, and uses these methods to explore the various predictions of reinforcement.

CHAPTER 2: FIGURES AND TABLES

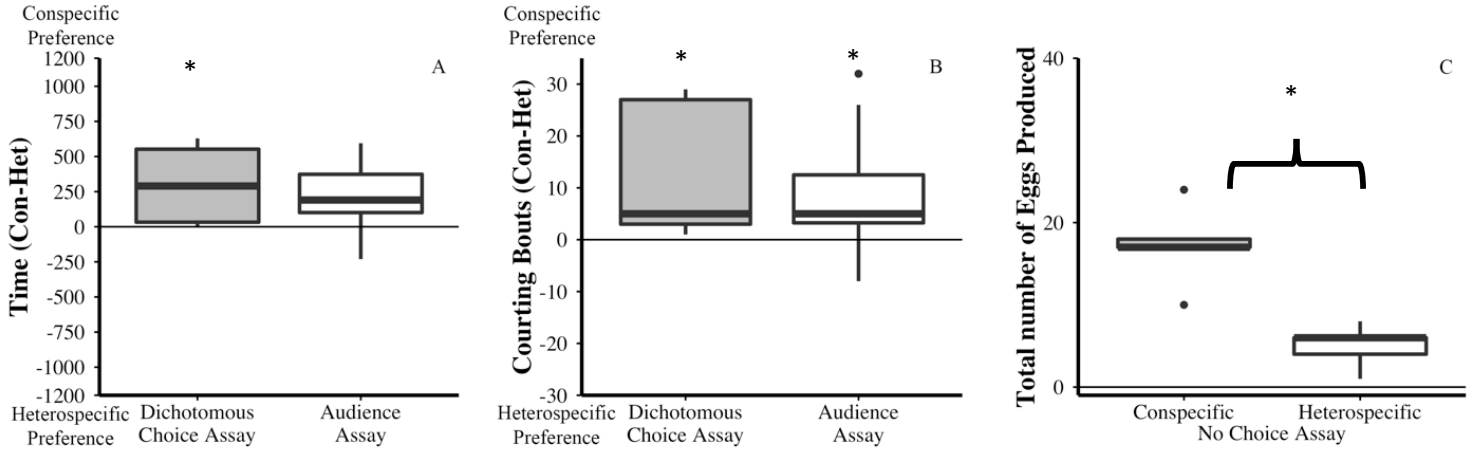

Figure 2.1: Boxplots indicating male mate preference for conspecific (positive numbers) or heterospecific (negative numbers) mates measured with A) the time metric, B) the courting bouts metric, or C) the total number of eggs produced metric. Asterisks (*) represent statistically significant preferences.

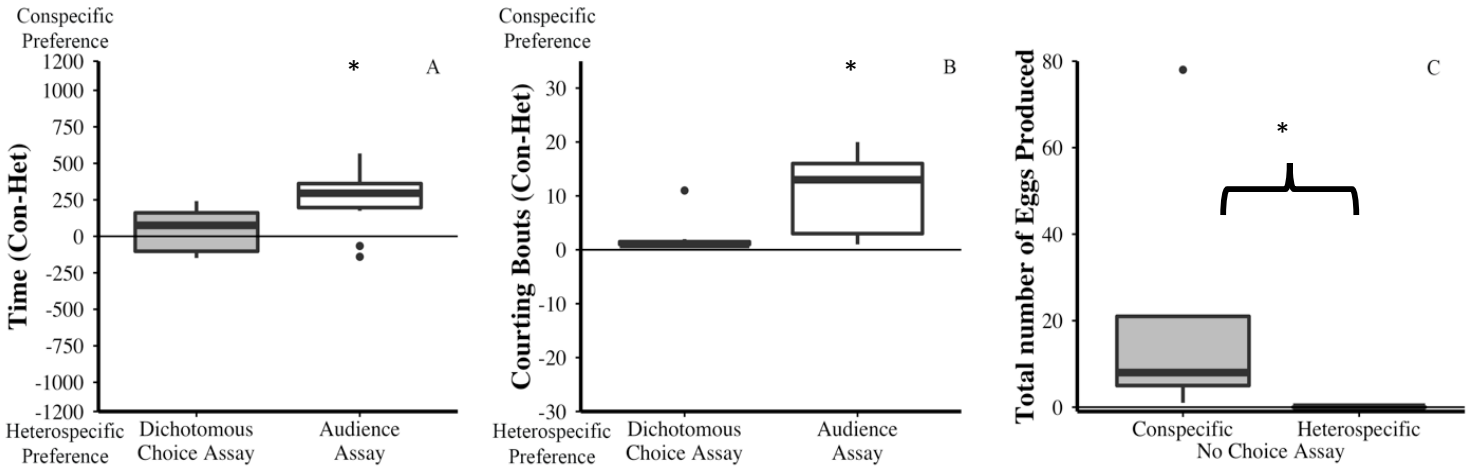

Figure 2.2: Boxplots indicating female mate preference for conspecific (positive numbers) or heterospecific (negative numbers) mates measured with A) the time metric, B) the courting bouts metric, or C) the total number of eggs produced metric. Asterisks (*) represent statistically significant preferences.

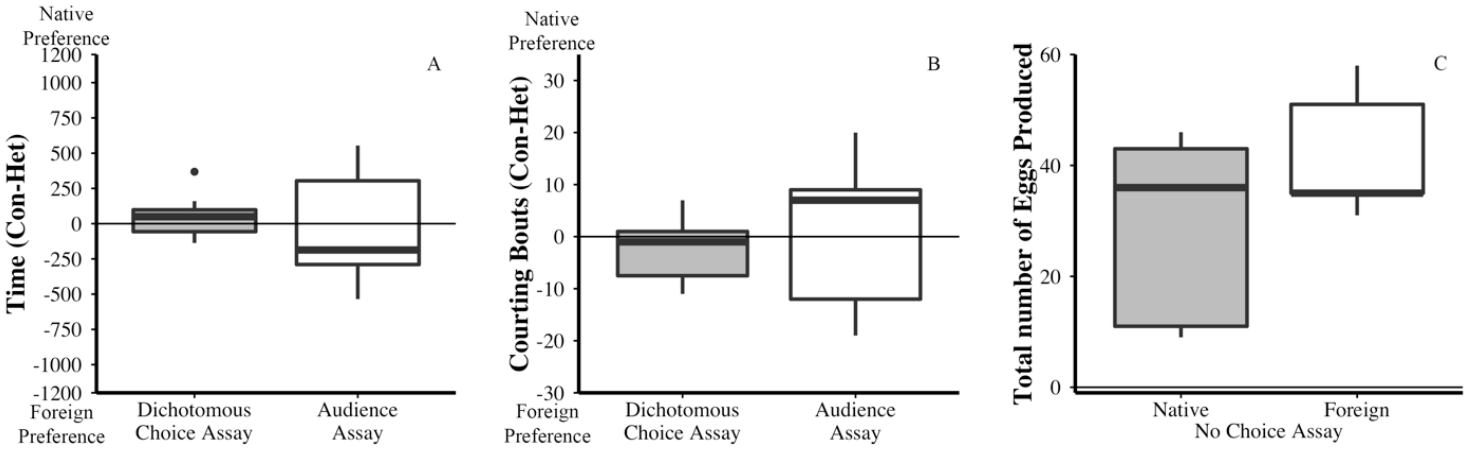

Figure 2.3: Boxplots indicating male mate preference for native (positive numbers) or foreign (negative numbers) mates measured with A) the time metric, B) the courting bouts metric, or C) the total number of eggs produced metric. Asterisks (*) represent statistically significant preferences.

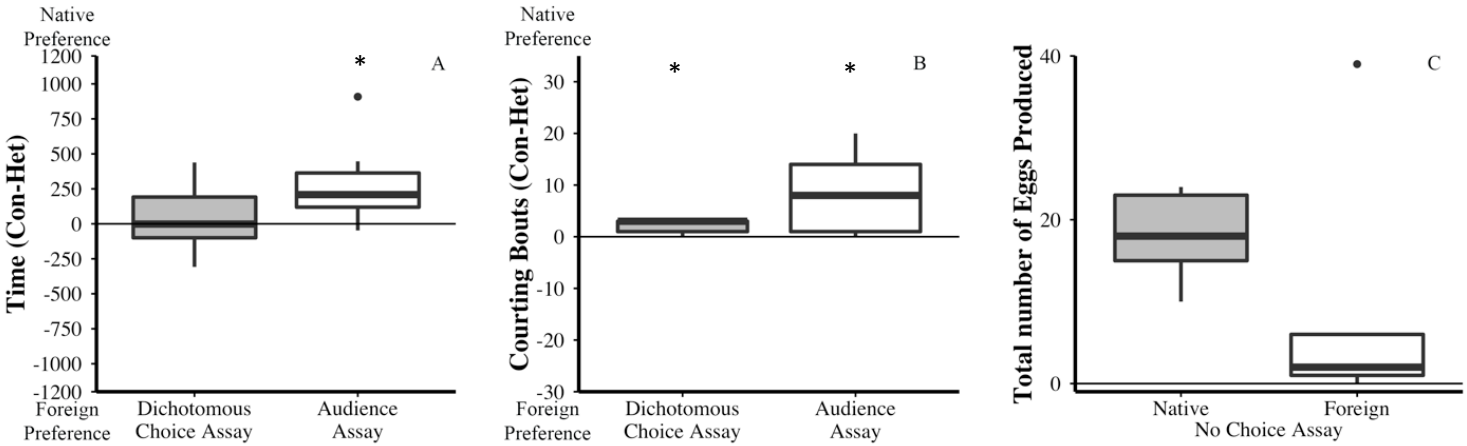

Figure 2.4: Boxplots indicating female mate preference for native (positive numbers) or foreign (negative numbers) mates measured with A) the time metric, B) the courting bouts metric, or C) the total number of eggs produced metric. Asterisks (*) represent statistically significant preferences.

Study	Assay Type	Sex	Metric	RI
Kozak et al. (2015)	Dichotomous Choice Assay	Male	Time	0.31
		Female	Time	0.44
Fuller et al. (2007)	No Choice Assay	Male	Eggs	0.44
		Female	Eggs	0.47

Table 2.1: Reproductive isolation (RI) values calculated using data from previous reinforcement studies in *L. goodei*.

Assay	Sex	Metric			
			N	RI	CI
Dichotomous Choice Assay	Male	Time	10	0.71	0.49, 0.93
		Courting	10	0.81	0.63, 0.99
	Female	Time	9	0.02	-0.36, 0.4
		Courting	7	1.00	1, 1
Audience Assay	Male	Time	10	0.51	0.11, 0.91
		Courting	10	0.55	0.11, 0.98
	Female	Time	10	0.50	0.15, 0.84
		Courting	9	0.95	0.87, 1.04
No Choice Assay	Male	Eggs	-	0.55	-0.13, 0.90
	Female	Eggs	-	1.00	1, 1

Table 2.2: Reproductive isolation (RI) values representing conspecific (positive numbers) or heterospecific (negative numbers) mate preference for male and female *L. goodei*. RI values were calculated for three metrics and three assays.

Assay	Sex	Metric	N	RI	CI
Dichotomous Choice Assay	Male	Time	9	0.04	-0.36, 0.45
		Courting	7	-0.31	-0.8, 0.18
	Female	Time	10	0.01	-0.35, 0.37
		Courting	7	0.66	0.22, 1.09
Audience Assay	Male	Time	9	0.05	-0.48, 0.58
		Courting	9	0.13	-0.39, 0.65
	Female	Time	10	0.64	0.39, 0.89
		Courting	9	0.89	0.63, 1.15
No Choice Assay	Male	Eggs	-	-0.18	-0.78, 0.51
	Female	Eggs	-	0.30	-0.49, 0.97

Table 2.3: Reproductive isolation (RI) values representing native (positive numbers) or foreign (negative numbers) mate preference for male and female *L. goodei*. RI values were calculated for three metrics and three assays.

CHAPTER 3: REINFORCEMENT AND CASCADE REINFORCEMENT IN THE LUCANIA SYSTEM: THE EFFECTS OF HETEROSPECIFIC PAIRINGS AND SEX ON MATE PREFERENCE

INTRODUCTION

The presence of reproductive isolation (RI) between groups is the backbone of the biological species concept (Mayr 1942). Typically, RI between groups arises as an incidental effect of selection on other ecologically relevant traits (Dobzhansky 1937; Coyne 2004). Only one type of selection, termed reinforcement, directly increases RI between groups. Reinforcement can occur if: 1) there is some level of post-zygotic isolation between diverging groups, 2) diverging groups are found in sympatry, and 3) diverging groups form unfit hybrids at low levels (Butlin 1987; Noor 1999; Servedio and Noor 2003). Selection against the production of unfit hybrids shifts mating traits and preferences away from phenotypes that previously overlapped with those of heterospecifics; thus completing the speciation process (Jaenike et al. 2006; Bewick and Dyer 2014; Pfennig 2016). Reinforcement not only completes speciation, but may also initiate it. If preferences or traits shift enough in sympatry, species may begin to discriminate against conspecifics from foreign populations. This increased preference for native mates in sympatry compared to allopatry is termed cascade reinforcement and can lead to speciation in the absence of post-zygotic isolation (Ortiz-Barrientos et al. 2009; Hoskin and Higgie 2010; Pfennig 2016). Despite the possible importance of reinforcement and cascade reinforcement, their pervasiveness to the speciation process is still largely unknown (Comeault and Matute 2016).

Understanding the role of reinforcement and cascade reinforcement in the speciation process requires documenting their frequency in nature. Fortunately, instances of reinforcement and cascade reinforcement can be identified by comparing mate preferences across populations.

Reinforcement increases conspecific mate preference in sympatry compared to allopatry, while cascade reinforcement increases native mate preference in the same manner, a pattern termed reproductive character displacement (Servedio 2004). Documenting patterns of reproductive character displacement has produced empirical evidence for reinforcement and cascade reinforcement (Kelly and Noor 1996; Noor 1999; Ortiz-Barrientos et al. 2009; Kozak et al. 2015; Comeault and Matute 2016). While most reinforcement and cascade reinforcement studies look for reproductive character displacement, they often exclude methodologies that could provide important information on how these processes are acting.

Two aspects of experimental design require close attention when documenting reproductive character displacement: 1) measuring mate preference in both sexes, and 2) measuring mate preference using heterospecific mates from both sympatric and allopatric populations. First, studies often document mate preferences using only one sex (Albert and Schluter 2004; Jaenike et al. 2006; Kronforst, Young, and Gilbert 2007; Marshall and Cooley 2000; Pfennig and Simovich 2002; but see: Kozak et al. 2015; Smadja and Ganem 2005). Failing to measure and compare mate preferences for both sexes (when appropriate) may lead to an over- or under-estimation of preference. Furthermore, if present, the difference in direction or strength of preferences between sexes may give insight to what factors drive or hinder reinforcement or cascade reinforcement. For example, the strength of reinforcement should correspond to hybrid fitness (Yukilevich 2012). Hybrid fitness, however, can be asymmetric between sexes or species (Pfennig and Simovich 2002; Jaenike et al. 2006; Yukilevich 2012). Measuring preference for both sexes can help determine which factors most affect hybrid fitness and consequently reinforcement and cascade reinforcement.

Second, reinforcement studies often standardize or ignore the sympatric or allopatric identity of heterospecific mates during experiments (Jaenike et al. 2006; Gregorio et al. 2012). An important step in identifying reinforcement is to measure and compare levels of RI between two species in sympatry and allopatry. A traditional method for measuring RI is to pair heterospecific species from sympatric populations and to pair heterospecific species from allopatric populations (Coyne and Orr 1989, 1997). Reinforcement may be acting if levels of RI are stronger between sympatric heterospecific pairings than allopatric heterospecific pairings. While these traditional pairings (sympatric mates always paired with sympatric mates, allopatric mates always paired with allopatric mates) can provide evidence for reinforcement, they may significantly overestimate levels of RI for sympatric groups. RI may appear high for sympatric groups who lack conspecific mate preference if they are paired with sympatric heterospecifics who presumably also exhibit conspecific mate preference. Pairing sympatric species with heterospecifics from allopatric populations (and vice versa), who presumably have weaker or non-existent mate preferences, may provide a more accurate measure of RI for species and sexes.

The primary goal of this study was to determine if using traditional pairings to measure RI overestimates levels of RI between sympatric groups. Specifically, I investigated this using the bluefin and rainwater killifish (Lucania goodei and Lucania parva). I measured RI for male and female sympatric L. goodei, allopatric L. goodei, sympatric L. parva, and allopatric L. parva in three ways. First, I measured RI using traditional pairs (pairs of L. goodei and L. parva from sympatric populations and pairs of L. goodei and L. parva from allopatric populations). Next, I measured RI for all four groups using only heterospecifics from allopatric populations. Here,

both allopatric and sympatric L. goodei were paired with allopatric L. parva, and vice versa. The advantage of this type of pairing is that individuals whom I presume have high levels of conspecific preference (sympatric populations) are matched with individuals that have lower levels of mate preference (allopatric populations). Finally, I measured RI using only heterospecifics from sympatric populations. These types of pairings allowed me to determine the extent to which conspecific preference (i.e. RI) is genuinely attributable to focal individual versus the partner with which it is interacting. Secondarily, I also sought to explore if conspecific or native mate preference varied between sexes. To accomplish this, I measured RI for males and females independently in the above experiment as well as when I measured native mate preference.

Methods

Study Organisms

The Lucania system is good for asking questions about reinforcement and cascade reinforcement because both processes have been documented in L. goodei and L. parva. Reinforcement in the Lucania system has extensive empirical support (Fuller et al. 2007; Berdan and Fuller 2012; Gregorio et al. 2012; Kozak et al. 2015), and evidence for cascade reinforcement is growing (Kozak et al. 2015; Chapter 2). Previous studies have also begun to document differences in mate preference between males and females for both species (Kozak et al. 2015; Chapter 2).

Collection and Care

During the summers of 2015 and 2016, I collected four types of populations for this experiment: 1) a sympatric population of L. goodei from Salt Springs (Marion County, FL), 2) sympatric populations of L. parva from California Creek (Dixie County, FL) and Salt Springs (Marion

County, FL), 3) an allopatric population of L. goodei from Blue Springs (Gilchrist County, FL), and 4) an allopatric population of L. parva from Lake Pontchartrain (St. Tammany County, LA). I used dip nets and seines to collected at least 10 males and 10 females from each of these populations. Using coolers, I transported the fish back to the University of Illinois where they resided for the duration of the experimental period. At the University of Illinois, fish were kept in large cattle tanks in an outdoor green house. Fish were exposed to natural light cycles and were fed a diet of brine shrimp and blood worms daily.

Assays

I used no-choice assays to measure mate preference for males and females from each of the four population types. I used total number of eggs produced by each pair as a proxy for preference. No-choice assays involved placing a single mate pair into a 38-liter tank for about 10 days. In addition to the two fish, tanks also included two top mops (yarn attached to a Styrofoam ball) and two bottom mops (yarn attached to PVC pipe) for spawning substrate. The first three days of the no-choice assay were used as acclimation time for the mating pair. Any eggs collected during this time were disregarded. During the remaining seven days, I collected and counted eggs from each mating pair. To cross all four population types, I ran my experiment for about five weeks in July and August of 2016.

I used the following numbers of males and females from each population type to determine mate preference:10 males and 10 females from Blue Springs to measure allopatric L. goodei preference;10 males and 10 females from Salt Springs to measure sympatric L. goodei preference; 6 males and 8 females from Lake Pontchartrain to measure allopatric L. parva

preference; and 11 males and 9 females from California Creek and 3 males and 3 females from Salt Springs to measure sympatric L. parva mate preference.

Experimental Design

I set up 40 aquaria in groups of 10, with each group containing 4 different types of males (a sympatric L. goodei male, an allopatric L. goodei male, an allopatric L. parva male, and a sympatric L. parva male) and 4 different types of females (a sympatric L. goodei female, an allopatric L. goodei female, an allopatric L. parva female, and a sympatric L. parva female). Immediately preceding the start of the experiment, I randomly assigned females to one of the four tanks in their group. Females remained in this tank for the entirety of the experiment. On week one, I randomly paired the males with a female in their group. I gave the pair three days to acclimate, and any eggs collected during this time were disregarded. The acclimation period was followed by seven days of egg collection from each pair. After egg collection on the seventh day, I removed males from their assigned tanks. I then randomly paired males with another female in their group and the process was repeated. I also followed this procedure for weeks three and four so that all males were paired for one week with all females. At the end of the experimental period, each group produced data for 16 unique male-female pairings (see Table 3.1 for all pair types). In total, my 10 replicates produced data for 160 unique pairings.

Unfortunately, I lacked sufficient numbers of allopatric L. parva from Lake Pontchartrain (6 males, 8 females). I had also hoped to use sympatric L. goodei and L. parva from the same location, but only had 3 males and 3 females of Salt Springs L. parva. To bolster my sample size and ensure that all animals experienced the same number of mates across replicate groups, I

supplemented L. parva from California Creek (a separate sympatric site) wherever there was a missing L. parva. Hence, all groups had male and female L. parva from a sympatric population, but two of the groups lacked an allopatric L. parva female and four lacked an allopatric L. parva male. This resulted in unequal sample sizes among the various pair types.

Data

I first used an ANOVA to determine if the total number of eggs produced varied between groups or between weeks of the experiment. I found no significant difference in the total number of eggs produced between groups or between weeks of the experiment, and therefore did not consider these effects further. I also asked whether there were differences in the number of eggs produced between the two populations that represented sympatric L. parva (Salt Springs and California Creek). I used a two sample T-test to investigate this, and found no difference between the two populations. I therefore pooled the two populations and simply considered them as sympatric L. parva. Finally, I asked if the sympatric L. parva individuals used as substitutes for allopatric L. parva differed in the number of eggs produced from the original sympatric L. parva. I explored this using a two-sample T-test, and found no difference between these two groups and included these individuals in the sympatric L. parva analysis. All analyses were performed in R (version 1.0.136).

Measuring Reproductive Isolation

The primary goal of my experiment was to compare estimates of RI from traditional mate pairings (sympatric with sympatric and allopatric with allopatric) to pairings where the heterospecifics were from allopatric populations, and to pairings where the heterospecifics were

from sympatric populations. To make these comparisons, I created a standardized formula to quantify RI. I used a variation of Stalker's isolation index (1942) with total number of eggs produced with a mate as a proxy for mate preference:

$$\frac{(\text{Total Eggs Produced with } L.\,goodei \text{ Mate}) - (\text{Total Eggs Produced with } L.\,parva \text{ Mate})}{(\text{Total Eggs Produced with } L.\,goodei \text{ Mate}) + (\text{Total Eggs Produced with } L.\,parva \text{ Mate})}$$

Using this formula, I first measured RI by comparing the number of eggs a group (i.e. male sympatric L. goodei, female allopatric L. parva, etc.) produced with conspecific mates (either L. goodei or L. parva) from their home population versus the number of eggs a group produced with heterospecific mates from an allopatric population (either L. goodei or L. parva). Next, I measured RI by comparing the number of eggs a group produced with conspecific mates from their home population versus the number of eggs a group produced with heterospecific mates from a sympatric population. Although all 16 types of pairs are represented in the dataset, they were not present in equal numbers. The unequal numbers prevented me from calculating RI values for each individual. Instead, I used a bootstrap resampling method to calculate RI and 95% confidence intervals. I calculated RI for each group for 5,000 replicates. RI values ranged from negative one to positive one, with positive numbers indicating L. goodei mate preference and negative numbers indicating L. parva mate preference. Preferences were considered significant if 95% confidence intervals did not overlap with zero. From these estimates, I created three graphs and tables to compare RI values from traditional pairings, pairings with heterospecific mates from allopatric populations, and pairings with heterospecific mates from sympatric populations.

Measuring Native Reproductive Isolation

I also asked if males and females from my four population types preferred native mates over foreign mates. I again used a variation of Stalker's isolation index (1942) to calculate native RI using the total number of eggs produced as a proxy for mate preference:

$$\frac{(\text{Total Eggs Produced with Native Mate}) - (\text{Total Eggs Produced with Foreign Mate})}{(\text{Total Eggs Produced with Native Mate}) + (\text{Total Eggs Produced with Foreign Mate})}$$

I calculated native RI for each group and considered conspecifics from the same population to be native mates and conspecifics from a different population to be foreign mates. For example, native RI for sympatric female L. goodei was calculated using total number of eggs produced with sympatric L. goodei males and total number of eggs produced with allopatric L. goodei males. I used a bootstrapping resampling method (5,000 replicates) to calculate RI and 95% confidence intervals for both sexes for each of my four population types. Again, RI ranged from negative one to positive one, but positive numbers indicated native mate preference and negative numbers indicated foreign mate preference regardless of the species.

Results

Reproductive Isolation using Traditional Pairings

When I measured RI values using traditional pairings, I found that sympatric populations of L. goodei and L. parva significantly preferred conspecific mates while allopatric populations showed no mate preference (Figure 3.1). L. goodei males and females had strong conspecific mate preference with RI values of about 0.97 (Table 3.2). The 95% confidence intervals for both male and female RI did not overlap with zero indicating that the preference was significant (Figure 3.1). L. parva males and female had similarly strong conspecific mate preference, -0.98

and -0.97 respectively (Table 3.2). The 95% confidence intervals for L. parva male and female RI values also did not overlap with zero, again indicating that these preferences were significant (Figure 3.1). RI values were very similar for males and females of both species, indicating that conspecific mate preference did not vary between sexes (Table 3.2). Allopatric populations of L. goodei showed moderately high RI values (0.59 and 0.55 respectively), but 95% confidence intervals for both males and females barely overlapped with zero (Figure 3.1). Allopatric L. parva males and females also lacked mate preference (Figure 3.1). Allopatric female L. parva RI was very similar to that of L. goodei at about -0.50 (Table 3.2). Male RI was slightly lower at -0.45 (Table 3.2). 95% confidence intervals for both males and females, however, significantly overlapped zero indicating no mate preference (Figure 3.1).

Reproductive Isolation with Heterospecifics from Allopatric Populations

When I used heterospecific mates from allopatric populations to calculate RI, I found a different pattern than that of the traditional pairings. I saw the biggest change in RI for male sympatric L. parva. Using traditional pairings, male sympatric L. parva RI values were -0.98, but here, they fell to -0.60 (Table 3.3). In contrast, the RI values for the remaining sympatric groups remained high (Table 3.3).

Reproductive Isolation with Heterospecifics from Sympatric Populations

When I used heterospecific mates from sympatric populations to calculate RI values I again found different patterns than what was seen for the traditional pairings. First, I found that the RI value for male allopatric L. goodei increased from 0.59 to 0.92 (Table 3.4). Furthermore, the 95% confidence interval for this RI shrunk significantly making this conspecific preference

statistically significant (Figure 3.3). Surprisingly, female allopatric L. goodei did not respond to sympatric mates in the same manner. Instead of increasing, RI values for female allopatric L. goodei fell from 0.55 to 0.26 (Table 3.4). The 95% confidence interval, however, still overlapped with zero indicating no significant preference for conspecifics or heterospecifics (Figure 3.3). Finally, I found that RI values for allopatric L. parva of both sexes increased from -0.50, females, and -0.45, males, to -0.97 and -0.98 respectively (Table 3.4). Their 95% confidence intervals also significantly shrunk making these preferences significant (Figure 3.3).

Native Reproductive Isolation

I found native mate preference in only one group: sympatric female L. parva (Figure 3.4). Overall, native RI values were much weaker than RI values for conspecific mate preference (Table 3.5). While sympatric conspecific RI values were close to negative or positive one, native RI did not exceed -0.38 or 0.56 (Table 3.5). Female sympatric L. parva exhibited significant native mate preference. RI values were moderately strong at 0.56 and the 95% confidence interval did not overlap with zero, indicating that the preference was statistically significant (Table 3.5). While no other groups showed a significant preference, I did find an interesting pattern in some allopatric groups. Although not significant, negative RI values for allopatric L. goodei (males and females) indicated a preference for foreign mates (Figure 3.4). The RI value for allopatric female L. parva was also negative, but not significant (Figure 3.4).

Discussion

Reproductive Isolation and Conspecific Mate Preference

My study recaptured the pattern of reinforcement that was previously documented in other studies using traditional mate pairings (Fuller et al. 2007; Gregorio et al. 2012; Kozak et al. 2015). RI values indicated significantly stronger conspecific mate preference in sympatric populations than in allopatric populations of the same species (Figure 3.1). These RI values, however, indicated that male and female L. parva and L. goodei all had similar levels of mate preference (~ ± 0.97) (Table 3.2). To determine if RI values for all sympatric groups were truly that high, I looked at the RI values calculated using only heterospecific mates from allopatric populations.

The informative comparisons come when RI is measured using heterospecifics from allopatric populations that presumably lack high levels of conspecific preference or may even vary in some critical trait. Now, if sympatric groups still display high RI, I am confident that the preference can be attributed to them and not their mating partner. These RI values revealed two important facts. First, male sympatric L. parva RI is much lower when paired with an allopatric heterospecific (-0.60) than when paired with a sympatric heterospecific (-0.98) (Figure 3.1 and 3.2). This indicates that the previously high RI values from traditional parings are at least partially due to the mate preferences or traits of the heterospecific mate. Second, I found that sympatric L. goodei of both sexes, as well as female sympatric L. parva maintained their high levels of RI even when paired with allopatric heterospecific mates. Taken together, these results suggest that male sympatric L. parva have the weakest conspecific mate preference of sympatric groups. This makes sense, as reinforcement predicts that the increase in pre-zygotic isolation should correspond to cost of hybridization (Yukilevich 2012). In the Lucania system, there is asymmetric fitness costs to hybridization between sexes, where hybrids produced from male L.

goodei and female L. parva parents suffer a significant reduction in fitness compared to hybrids formed from female L. goodei and male L. parva (Fuller 2008a). In addition to the asymmetric costs of hybridization, I previously found that female L. goodei mate preference may also be increased due to their energetic investment in reproduction (production of eggs) (Chapter 2). My data here agrees with both ideas. Male sympatric L. parva have the least to lose during hybrid pairings and their level of RI, when paired with allopatric heterospecific mates, reflects this (Figure 3.2).

The idea that the levels of RI vary due to whether the heterospecific originates from a sympatric or allopatric population can also be found when RI is measured using only heterospecifics from sympatric populations (Figure 3.3). Here, RI values are inflated for individuals from allopatric populations. When paired with allopatric heterospecifics, male allopatric L. goodei and allopatric L. parva of both sexes displayed no conspecific mate preference, but when paired with sympatric heterospecifics these groups suddenly displayed high conspecific mate preference (Figure 3.3). This discrepancy indicates that these high RI values are inflated due to the conspecific mate preference of the sympatric heterospecific mate, and not due to any preference from the allopatric groups.

Native Mate Preference

Finally, female sympatric L. parva were the only group to prefer native mates over foreign mates (Figure 3.4). This supports previous work in L. parva, where females from sympatric populations preferred mates from their native population over mates from foreign populations (Kozak et al. 2015). In previous work, I found that female L. goodei may also prefer native mates over foreign

mates (Chapter 2). This preference, however, was only detected using choice assays and was not detected using no-choice assays. My results from this study therefore support my previous work, if only due to the conservative nature of no-choice assays.

In conclusion, my results support reinforcement in the Lucania system. Furthermore, I highlight the importance of using heterospecific mates from both sympatric and allopatric populations when testing for reinforcement. I propose that reinforcement studies that do not do this may overestimate RI values for sympatric groups. By testing groups with all other population types, I found that male sympatric L. parva have significantly weaker conspecific mate preferences than any other sympatric group. Finally, I found mate preferences consistent with cascade reinforcement in female L. parva. Whether or not these preferences can initiate or complete speciation remains to be determined.

CHAPTER 3: FIGURES AND TABLES

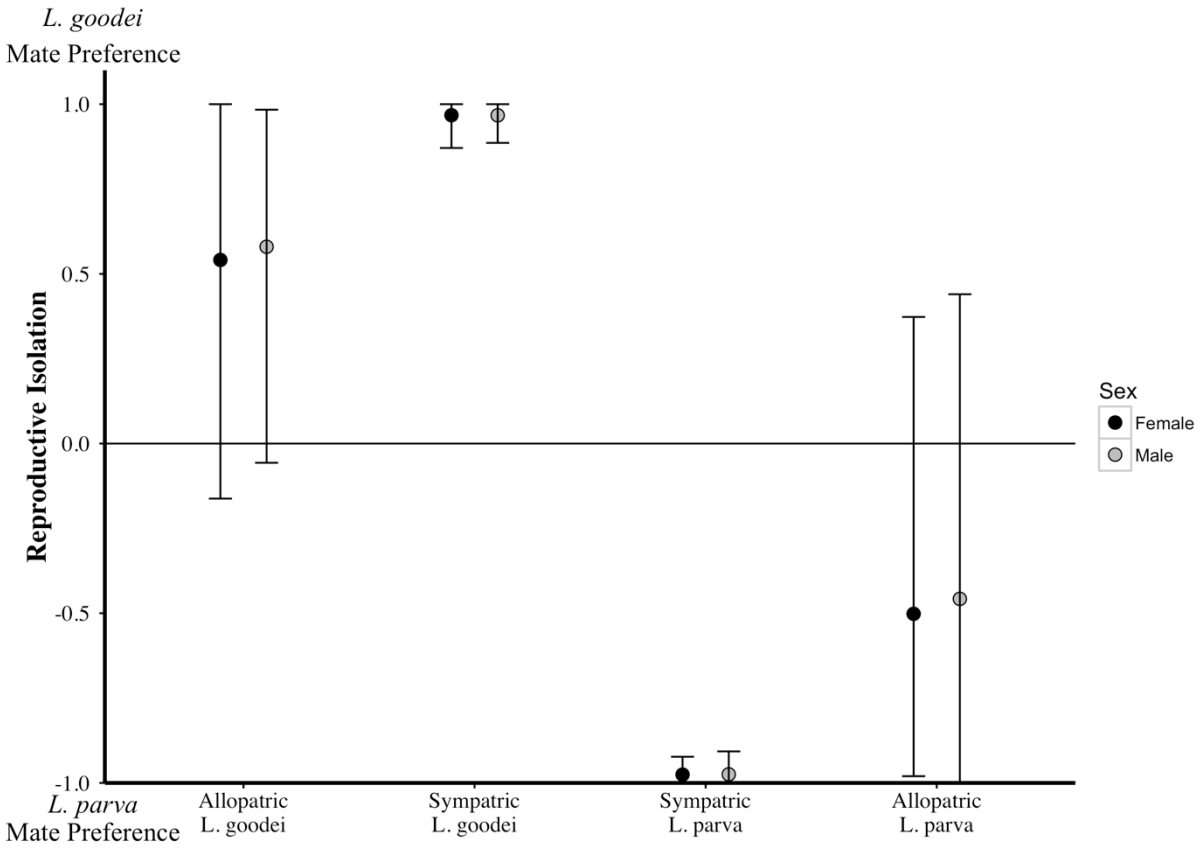

Figure 3.1: Conspecific mate preference for male and female *L. goodei* and *L. parva* from sympatric and allopatric populations measured using traditional pairings.

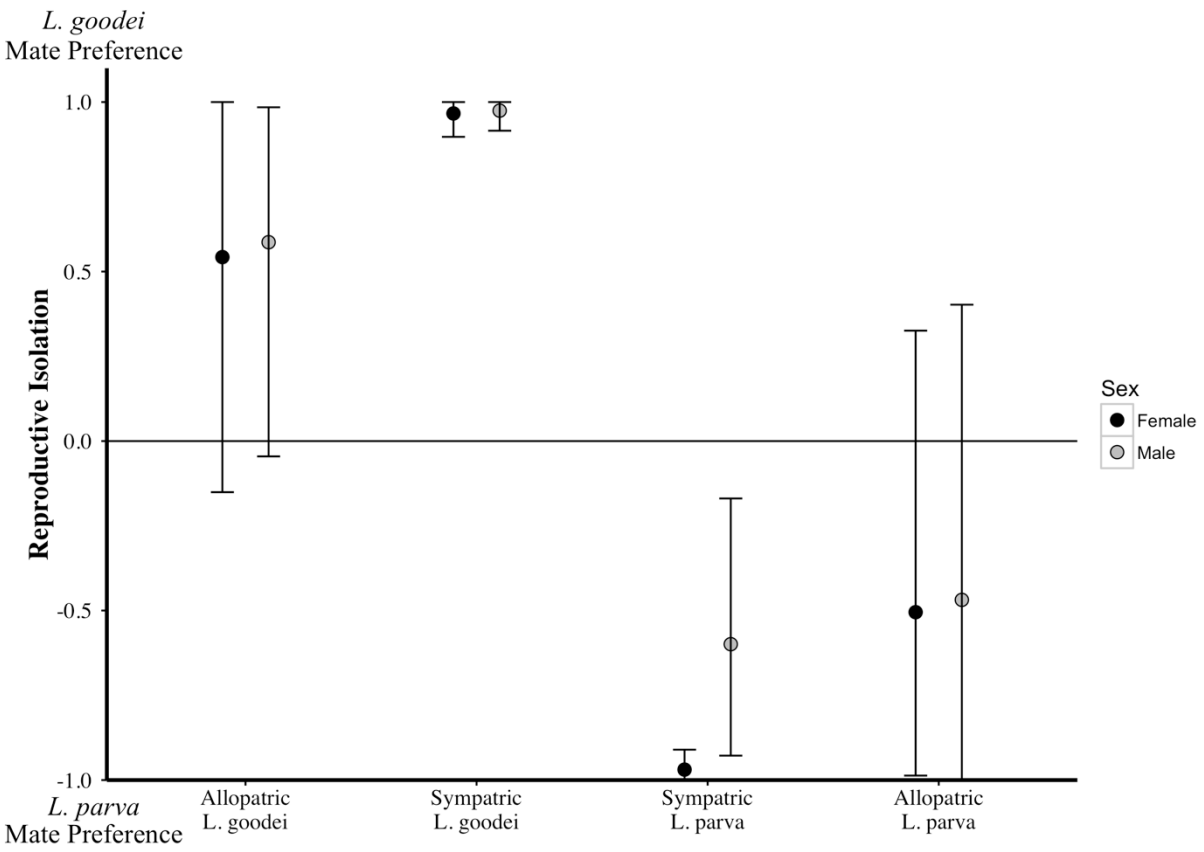

Figure 3.2: Conspecific mate preference for male and female *L. goodei* and *L. parva* from sympatric and allopatric populations. RI values were calculated using heterospecific mates only from allopatric populations.

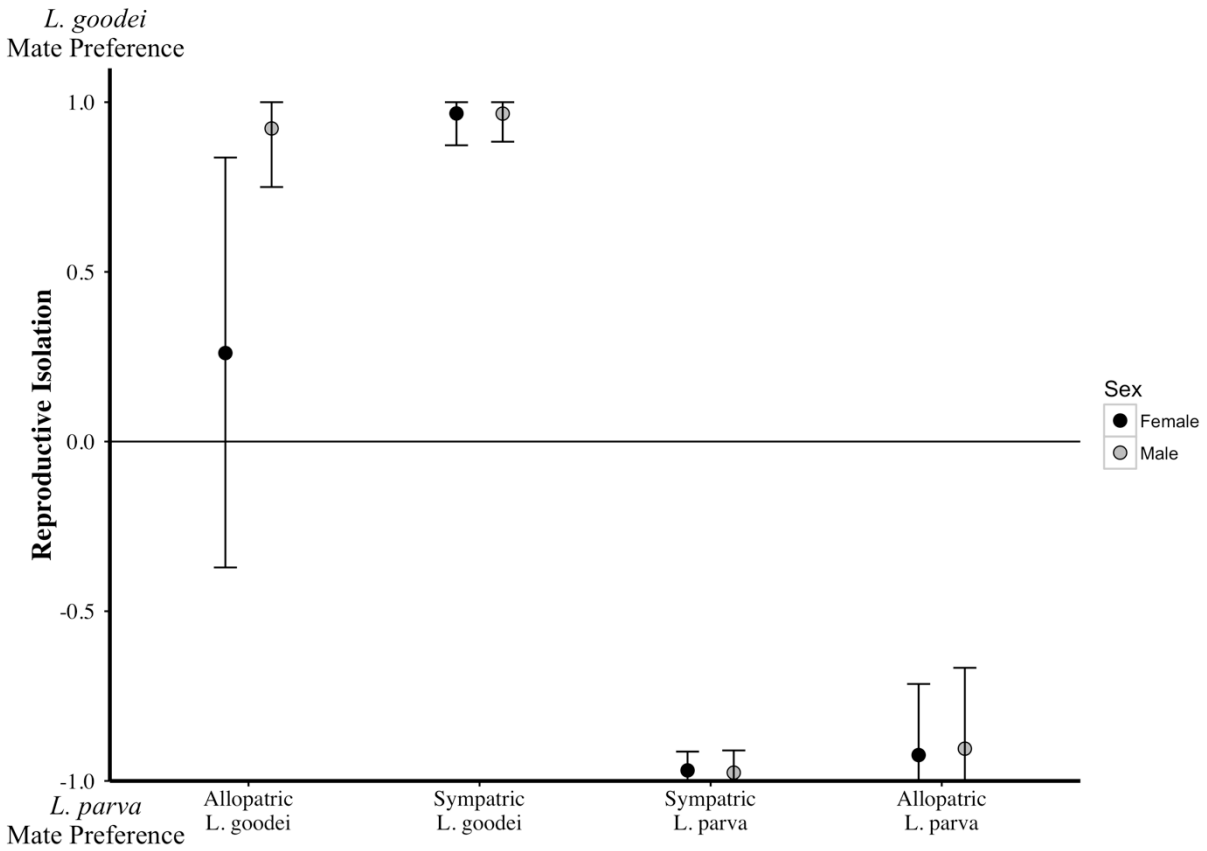

Figure 3.3: Conspecific mate preference for male and female *L. goodei* and *L. parva* from sympatric and allopatric populations. RI values were calculated using heterospecific mates only from sympatric populations.

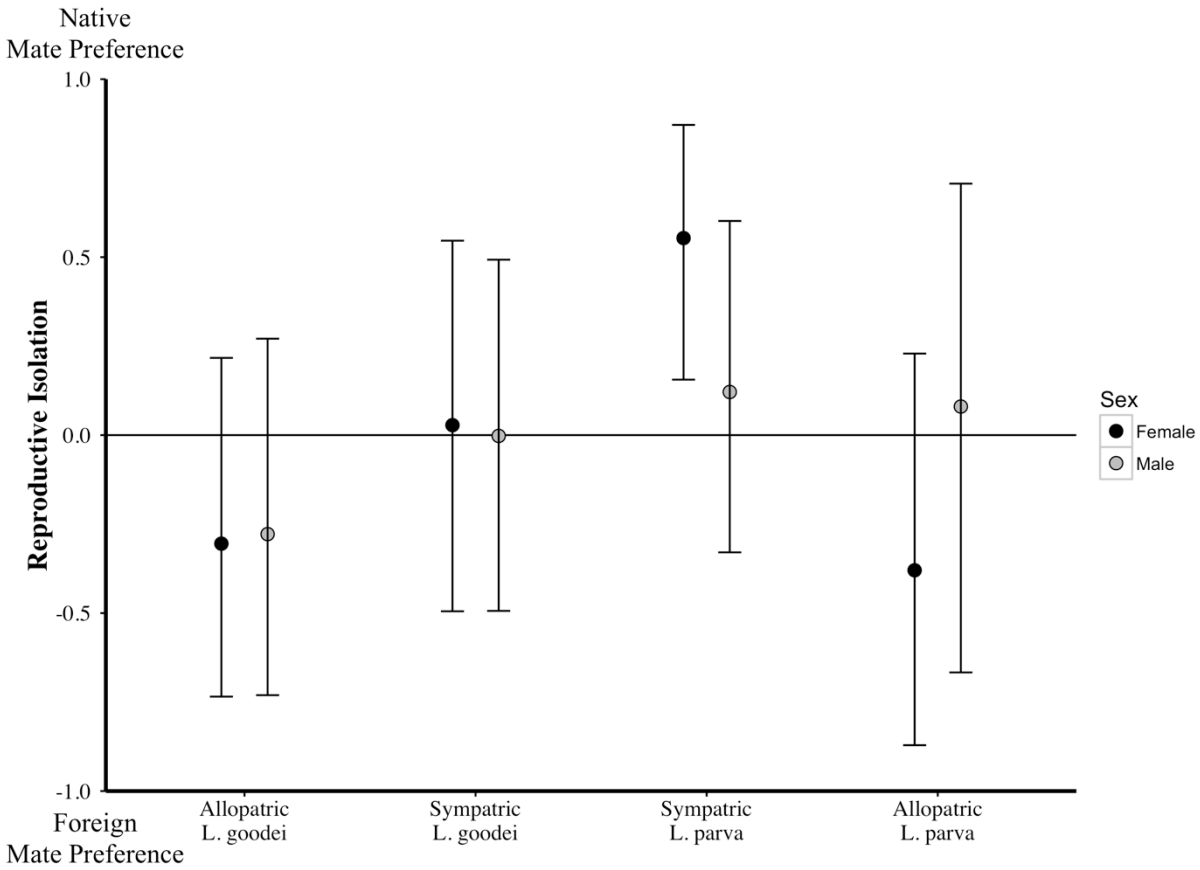

Figure 3.4: Native or foreign mate preference for male and female *L. goodei* and *L. parva* from sympatric and allopatric populations. RI values were calculated using total number of eggs produced by conspecific mates from native populations and foreign populations.

Female	Male	N	Mean Number of Eggs Produced
Allopatric *L. goodei*	Allopatric *L. goodei*	10	14.20
	Sympatric *L. goodei*	10	27.50
	Allopatric *L. parva*	6	7.17
	Sympatric *L. parva*	14	5.57
Sympatric *L. goodei*	Allopatric *L. goodei*	10	24.80
	Sympatric *L. goodei*	10	27.30
	Allopatric *L. parva*	6	0.67
	Sympatric *L. parva*	14	0.29
Allopatric *L. parva*	Allopatric *L. goodei*	8	4.50
	Sympatric *L. goodei*	8	0.38
	Allopatric *L. parva*	6	20.00
	Sympatric *L. parva*	10	27.70
Sympatric *L. parva*	Allopatric *L. goodei*	12	0.42
	Sympatric *L. goodei*	12	0.33
	Allopatric *L. parva*	6	16.00
	Sympatric *L. parva*	18	19.11

Table 3.1: List of the16 unique mate pair types. Table also includes the number of pairs in each type as well as the mean number of eggs produced by said pair type.

Species	Population	Focal Sex	RI	CI
L. goodei	Allopatric	Female	0.55	-0.14, 1
		Male	0.59	-0.03, 0.99
	Sympatric	Female	0.97	0.86, 1
		Male	0.97	0.89, 1
L. parva	Allopatric	Female	-0.50	-0.98, 0.39
		Male	-0.45	-1, 0.54
	Sympatric	Female	-0.97	-1, -0.92
		Male	-0.98	-1, -0.91

Table 3.2: Conspecific mate preference for *L. goodei* and *L. parva* males and females from sympatric and allopatric populations. RI values were calculated using traditional pairings (i.e. the total number of eggs produced with a conspecific and heterospecific from the same population type, sympatric or allopatric, as the focal group).

Species	Population	Focal Sex	RI	CI
L. goodei	Allopatric	Female	0.55	-0.14, 1
		Male	0.59	-0.03, 0.99
	Sympatric	Female	0.97	0.9, 1
		Male	0.97	0.91, 1
L. parva	Allopatric	Female	-0.50	-0.98, 0.39
		Male	-0.45	-1, 0.54
	Sympatric	Female	-0.97	-1, -0.91
		Male	-0.60	-0.93, -0.19

Table 3.3: Conspecific mate preference for *L. goodei* and *L. parva* males and females from sympatric and allopatric populations. RI values were calculated using the total number of eggs produced with a conspecific from the focal group's home population and the total number of eggs produced with a heterospecific only from allopatric populations

Species	Population	Focal Sex	RI	CI
L. goodei	Allopatric	Female	0.26	-0.37, 0.85
		Male	0.92	0.76, 1
	Sympatric	Female	0.97	0.86, 1
		Male	0.97	0.89, 1
L. parva	Allopatric	Female	-0.92	-1, -0.7
		Male	-0.90	-1, -0.65
	Sympatric	Female	-0.97	-1, -0.92
		Male	-0.98	-1, -0.91

Table 3.4: Conspecific mate preference for *L. goodei* and *L. parva* males and females from sympatric and allopatric populations. RI values were calculated using the total number of eggs produced with a conspecific from the focal group's home population and the total number of eggs produced with a heterospecific only from sympatric populations

Species	Population	Focal Sex	RI	CI
L. goodei	Allopatric	Female	-0.31	-0.73, 0.22
		Male	-0.25	-0.71, 0.32
	Sympatric	Female	0.05	-0.48, 0.57
		Male	-0.01	-0.53, 0.49
L. parva	Allopatric	Female	-0.38	-0.87, 0.22
		Male	0.07	-0.68, 0.7
	Sympatric	Female	0.56	0.15, 0.87
		Male	0.12	-0.35, 0.59

Table 3.5: Native mate preference for *L. goodei and L. parva* males and females from sympatric and allopatric populations. RI values were calculated using the total number of eggs produced with a conspecific from the focal group's native population and a conspecific from a foreign population.

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